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Original Article

Association of water stress and Fusarium solani exacerbated Dalbergia sissoo dieback disease

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Abstract

Globally, there is a growing concern about tree mortality due to harsh climates and changes in pest and disease patterns. However, experimental studies on the interactions between biotic and abiotic stresses in plants are relatively scarce. In this study, we investigated the interaction between Fusarium solani and water-stressed Dalbergia sissoo saplings. We postulated that under drought conditions, sissoo plants would become more susceptible to dieback infestation. Five fungi, including Fusarium oxysporum, Curvularia lunata, Cladophialophora carrionii, Alternaria alternaria, and Fusarium solani, were isolated from an old shisham tree showing advanced symptoms of dieback infestation. These fungi were identified based on their ITS sequence homology and spore characteristics. Dieback development was more pronounced in plants experiencing water stress, regardless of their predisposition or whether it occurred simultaneously. Lesions were more noticeable and longer in predisposed saplings (3.8cm), followed by simultaneous (2.4cm) and much smaller lesions in seedlings that were inoculated and well-watered (0.24cm). Progressive browning of the upper leaves, which lowers sapling height in predisposed, simultaneous, and well-watered inoculated saplings to 8.09 inches, 5.93 inches, and 17.42 inches, are typical dieback symptoms. Water stress causes the loss of chlorophyll a, b, and carotenoids, which reduces stomatal conductance, transpiration rate, and photosynthetic activity, leading to poor development and mortality. Similarly, predisposed, simultaneous, and well-watered inoculated seedlings expressed increased activity of CAT (22.57, 18.148, and 9.714 U/mg) and POD (3.0, 4.848, 1.246 U/mg), to reduce the damage caused by elevated levels of H2O2 expression. It is concluded that water stress is the main cause of dieback in shisham saplings that subsequently disposed of infected seedlings to secondary agents such as fungi and insects in the advanced stages of the dieback with prolonged drought stress. The lack of dieback in native populations is attributed to the absence of several ecological stresses, including water stress, extended droughts, waterlogging, and salinity. This study emphasizes the need for additional research into the effects of abiotic factors linked with fungal diseases on the long-term production and management of D. sissoo in Pakistan.

Keywords: Dalbergia sissoo, Drought, ITS sequences, Fusarium solani, Physiological and Biochemical responses.

1. Introduction

Tree mortality caused by climate change has increased globally and in Pakistan [1]. While most of the research has indicated that heat and/or drought are the main causes of tree death, tree death is a complicated process brought on by several interacting biotic and abiotic factors, ranging from climatic stress sequences to stressor-driven changes in plant, insect, and pathogen dynamics to disease infection stand life histories [2]. According to Sturrock et al [3], plants can generally recover depending on the duration and intensity of the stress unless they are also affected by contributing factors such as pests and diseases. Long-term

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changes in humidity, rainfall, or soil characteristics are all risk factors. Short-term variables such as drought/heat or defoliating insects are examples of driving factors.

Dalbergia sissoo, locally known as "Shisham or Talli" is native to tropical and subtropical Himalayan regions of Pakistan, India, Nepal, Bhutan, Bangladesh, and Afghanistan [4]. It has been introduced to other countries around the world, most notably in America, Asia, Africa, and Australasia [1, 5-6]. Similarly, it was taken to different ecological regions of Pakistan in 1880, primarily to meet the need for fuelwood [4]. It is one of the most important and widely planted tree species in Pakistan for agroforestry, fuel, lumber, and soil binding. Its wood is highly valued for its exceptional tensile strength, color, texture, and other physio-mechanical properties [1,4]. Wood is also valued for heating and cooking due to its high calorific value. Its wood produced raw material for a variety of wood-based industries, particularly furniture and housing.

Despite the *D. sissoo* being native to Pakistan, serious issues with plantation establishment are expected, particularly in non-native regions of Pakistan [7]. Dieback caused widespread shisham mortality in 1998, particularly on artificial plantations, roadsides (20 – 40%), canals (75 – 80%), and farmlands (10%) in KPK, Punjab, and Sindh. The primary causes of shisham dieback are unknown, but it is widely assumed that fungi are the main cause of dieback [7,8]. *Fusarium solani* has been frequently isolated from various parts of dieback-infected sissoo plants and is reported to be a common endophyte in *D. sissoo* [7]. *F. solani* is a genus of at least 26 filamentous fungi in the Nectriaceae family's Ascomycota division. It is a soil-borne fungus that causes disease in a variety of plant species [9].

Several factors influence climate change, including drought, high temperatures, and flooding, all of which have an impact on plant health. As a result, understanding these factors as the outcomes of interactions on plant health is critical for forecasting global change [10]. Droughts are becoming more common and severe, reducing the time plants must respond. Plant responses to water scarcity have been discovered to be dynamic, interactive, long-term, time-dependent, and stress-dependent [11]. Water scarcity impacts plants physiologically, biochemically, and molecularly [12]. *D. sissoo's* processes and dynamics, as well as its interactions with biotic variables such as fungal diseases, are unknown. Pathogen outbreaks also modify plant physiological systems, such as primary and secondary metabolisms linked to defense mechanism activation, which affects plant growth and development [13]. Plant-pathogen interactions can have different outcomes depending on the history of abiotic stress experienced by plants during their life cycle.

Water scarcity can alter a plant's physiological status by making it more susceptible to abiotic factors and encouraging fungal attacks [14]. The relationship between abiotic stress and disease susceptibility in plants is well established [15-16], and thus, a better understanding of these variables is critical for disease management [3]. When compared to non-predisposed plants, predisposed plants can generate a faster and more powerful expression of cellular defense responses in a future stress experience [17-18]. Although reactive oxygen species (ROS) formation has been identified as a potential plant defense mechanism that may improve infection resistance [19]. Recently, Miletić et al. [20] asserted the relationship between droughts and pests in Norway Spruce as the main causes of dieback outbreaks. Nonetheless, most pathogenicity studies failed to identify *F. solani* as the primary cause of the dieback, and little is known about the role of water stress in the development of the shisham dieback. This study aims to evaluate the role of water stress in association with *F. solani* by assessing infection rate, disease intensity, plant growth, and biochemical and physiological responses of shisham seedlings under combined stresses.

2. Materials and methods

2.1. Experimental design

Open-pollinated seeds were collected at random from various shisham mother trees in Basham, a town situated in Pakistan's Himalayan Khyber Pakhtunkhwa (KPK) province. This region is one of the *D. sissoo's* places of origin. The climate is mainly subtropical, with heavy rainfall, high temperatures, and humidity. The soil is sandy loam with good drainage. *D. sissoo* pods were soaked overnight in water at room temperature before being planted in plastic pots filled with sandy loam soil. Saplings were watered every day until they were 6 months old. The experiment included two treatments, as explained in Fig. 1. In the first treatment, all plants were watered at 85% field capacity, where control plants were inoculated with a sterile PDA (mock), and a second set of plants were inoculated with *F. solani*. In the case of the second treatment, all plants were maintained at 25% field capacity under water-stressed conditions, where one set of plants was inoculated with *F. solani* and maintained at the 25% field capacity, whereas the second set of plants was predisposed to 25% field capacity for two weeks before inoculated with *F. solani* (Fig. 1). The control plants were subjected to 25% field capacity without inoculation. Ten seedlings replicated each treatment, and the entire experiment was designed in a completely randomized design (CRD). Historical climate data for Pakistan (Fig. 2) on mean annual rainfall (mm) and temperature (^{O}C) were obtained from (https://climateknowledgeportal.worldbank.org/download-data).

2.2. Pathogen isolation

Shisham trees exhibiting the characteristics of dieback symptoms were identified on the Campus of the University of Agriculture Faisalabad, Punjab, Pakistan. Brown-colored xylem tissues were removed for fungal isolation. The xylem tissues were cleaned with tap water and then with sterile water for further processing. Tissue samples were immersed in absolute ethanol for one minute before being rinsed with sterile water and dried on sterile blotting paper. Five sterile tissues were cultured on potato dextrose

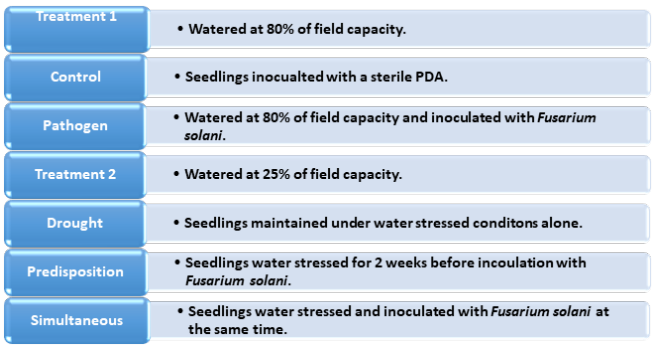

Fig. 1. Schematic diagram of the experiment design used in this study.

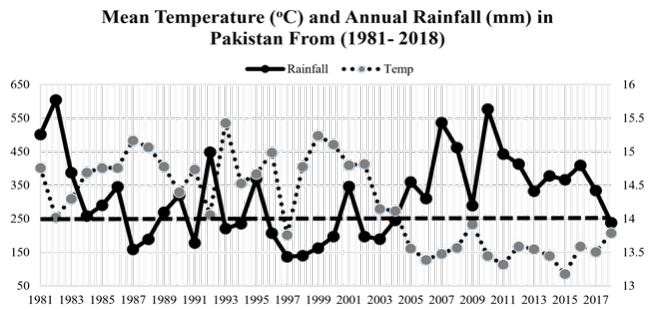

Fig. 2. Historical data on changes in Pakistan's rainfall and temperature patterns from 1981 to 2018. Between 1995 and 2000, there was a significant decrease in annual rainfall, with a mean rainfall of less than 200 mm and a high mean temperature increase of more than 14 degrees Celsius. This period coincides with the shisham dieback epidemic in Pakistan, and the subsequent increase in mean rainfall resulted in the dieback being reduced [1].

agar (PDA). The cultures were incubated at 25°C for one week. Fungi were cultured and subsequently identified based on both conidial characteristics and nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker.

2.3. Internal transcribed sequence region (ITS) DNA barcoding for fungal identification

Total genomic DNA was extracted from one-week-old mycelia using the modified Al Samarrai and Schmid protocol [21]. The quality of the extracted DNA was determined by running it on 1% agarose gel and stained with ethidium bromide before being subjected to PCR amplification. To amplify fungal DNA, the primers ITS1F (5'- CTTGGTCATTTAGGAAGTAA-3') and ITS4R (5'- TCCTCCGCTTATTGATATGC-3') were employed, which are designed to bind the internal transcribed spacers (ITS) of nuclear rDNA (Op de Beeck et al. [22]. PCR reactions were performed in a total volume of 25 µL with a final concentration of 1 x buffer (Invitrogen, Carlsbad, CA, USA), 0.2 mM each dNTP (Invitrogen, Carlsbad, CA, USA), 1.5 mM $MgCl_2$ (Invitrogen, Carlsbad, CA, USA), 0.2 U/mL of Platinum Taq polymerase (Invitrogen, Carlsbad, CA, USA), 1 µM each forward and reverse primer, and 3 µl template DNA. PCR reactions were performed at 94°C for 3 min in a C1000 Touch Thermal Cycler (Bio-Rad Laboratories Inc., UK), followed by 35 cycles, starting with denaturation (30 s at 94°C), annealing (30 s at 55°C) and extension (1 min at 72°C), followed by 10 min extension at 72°C. PCR amplicons were visualized on 1.0% agarose gel electrophoresis. To ensure fungal amplicon specificity, the anticipated length of PCR amplicons was compared to a DNA ladder. All amplicons were sequenced from both ends, and the sequencing chromatogram for each strand was verified for mismatches before being combined into a consensus DNA barcode using BioEdit ver. 7.0.4 (20). The BLASTn was used to screen the NCBI nucleotide database for the closest homologs, resulting in the identification of fungal species.

2.4. Morphological identification

F. solani, isolates matched with ITS sequences in Gene Bank were further validated using spore morphology. A small mass of the fungal culture was removed from the PDA plate surface and placed on a glass slide. Following the Fusarium manual, the conidial characteristics were recorded and identified based on the shape of micro and macroconidia [23].

2.5. Pathogenicity testing in greenhouse

Pathogenicity testing was performed in a greenhouse on six-month-old seedlings. Before sapling inoculation, the stem surface was sterilized with 70% ethanol. To remove the bark and expose the cambium, a shallow "I" shaped cut was made at the seedling's base (5 cm above the soil surface) with a sterile scalpel. Jung and Nechwatal [24] put a 5 mm mycelial plug into the incision confronting the wound. Similarly, control plants were administered sterile PDA plugs. To confirm Koch's hypotheses, the fungus was retrieved from each infected plant two months later. After removing the wood tissues from the lesions' borders, they were cleansed with 5% hypochlorite for two minutes, then 70% ethanol for one minute, rinsed with sterile distilled water, dried on sterile filter paper, and plated on PDA media. Additionally, seedlings infected with PDA were re-isolated.

2.6. Field screening in hot spot

One-year-old root shoot cuttings were planted at Cheechawatni and Abassia Plantations in Punjab, Pakistan, to study dieback incidence under natural field conditions. A dieback "Hot Spot" is a field that has previously been infected by dieback. The Cheechawatni plantation is in Punjab, Pakistan, located at an elevation of 153.6 to 163.7 m above sea level. The annual rainfall averages 200-250 mm, and the *D. sissoo* populations were severely damaged by dieback, which resulted in 99 percent tree mortality in 1998. The Abbassia plantation is in Bahawalpur, in the southeastern part of the Punjab. The plantation is irrigated by Melsi Link Canal that runs through the plantation, and its water is available all year. The canal also helps maintain the water table on the plantation. Annual rainfall ranges from 200 to 250 mm. The field chosen for *D. sissoo* along the canal had no previous shisham planting history. On both plantations, 2,000 one-year-old plants were planted at a 5 x 5 ft spacing and watered with canal water. In June 2019, 200 plants on both plantations were artificially inoculated with *F. solani* using a method proposed by Jung and Nechwatal [24].

2.7. Plant monitoring

Plants were monitored regularly, and information on external symptoms, such as leaf color changes and wilting, was collected. After two months of treatment, data on growth indices, disease incidence, and physiological and biochemical characteristics were obtained.

2.8. Growth parameters (inch)

Plant height was measured twice, once on the day of inoculation and secondly on the final day of the trial. The growth rate was computed by subtracting the plant's initial and final heights.

2.9. Lesion growth (cm)

The cut length was measured longitudinally on the day of the inoculation. After two months, the growth of lesions (cm) in infected seedlings was measured. The bark was removed from the inoculated site and the lesion's growth along the xylem was measured. The lesion growth (%)

was obtained by subtracting the final length from the initial cut length.

2.10. Osmotic potential

Fresh leaf tissue was collected and stored at 0°C for 3–4 days. The tissue was thawed at room temperature for 30–45 min before draining the cell sap from the leaves. To measure leaf osmotic potential, an osmometer was filled with 50 µL of cell fluid [23]. Leaf water potential was measured at 6:40 AM using a pressure chamber (Plant Moisture Stress, PMS Instruments) in five replicates for each sapling [24]. Photosynthetic rate, stomatal conductance, water use efficiency, and transpiration rate were calculated using a portable IRGA (Infrared Gas Analyzer, Li 6200, Licor). Measurements were taken at noon time on five randomly selected leaves for each sapling.

2.11. Photosynthetic rate, Transpiration Rate, Stomatal conductance, Water use efficiency

IRGA portable equipment was utilized to analyze photosynthetic rate (µmol m-2 s-1), stomatal conductance (mmol m-2 s-1), water usage efficiency, and transpiration rate (mg H2O m-2 s-1) (Infra-Red Gas Analyzer, Li 6200, Licor). Measurements were taken around midday on five randomly selected leaves for each treatment.

2.12.Chlorophyll contents

Chlorophyll was measured by collecting five leaves at random from each plant and taking five readings per leaf. A 0.5 g f. wt. leaf mass was crushed to powder, and 2 ml of 80 percent acetone was added before homogenizing the sample. The mixture was centrifuged for 5 minutes at 15000 rpm, and the supernatant was used to calculate chlorophyll using the NanoDrop Spectrophotometer (NanoDropTM). The chlorophyll content was measured at 663 nm absorbance. The same method was used to determine chlorophyll b and carotenoids, with absorbance measurements taken at 645 nm and 480 nm, respectively [25].

2.13 Pigment determination

Sims and Gamon's technique [26] was followed to extract photosynthetic pigments using cold acetone and 50 mM Tris buffer pH 7.8 (80:20 v:v). The readings were acquired with a PerkinElmer Spectrophotometer at wavelengths of 470, 537, 647, and 663 nm. Sims and Gamon's [26] formulas were used to calculate the concentrations of total chlorophyll, chlorophyll a (Chla), b (Chlb), and carotenoids.

2.14. Estimation of hydrogen peroxide (H_2O_2)

The method for collecting and detecting hydrogen peroxide (H_2O_2) from *D. sissoo* leaves was adapted from Velikove et al. [27]. Leaf tissues were frozen using liquid nitrogen and ground with 5 mL of 0.1 percent (w/v) trichloroacetic acid. The mixture was centrifuged at 15.000 x g for 15 minutes at 4°C to separate the phases. To 0.5 ml of supernatant, add 1 mL of 1 M KI and 0.5 mL of 10 mM K_2HPO_4 (pH 7.0) buffer. The reaction mixture was left in the dark for 1 hour, and the absorbance was measured at 390 nm. H_2O_2 was calculated using a standard curve.

2.15. Antioxidant enzyme activity determination

For enzymatic activities, 1.0 g of fresh leaf was macerated in 25 mL of cold extraction buffer with 0.5 g of PVPP. We utilized a 50 mM sodium phosphate buffer (pH 7.0) to test CAT and a 100 mM sodium phosphate buffer (pH 6.4) to determine peroxidase. Homogenates were removed by centrifugation at 27.000 x g for 50 minutes at 4 °C, and the supernatants were used to determine enzyme activity [28]. Beers and Sizer's (1952) approach was used to quantify CAT activity. The CAT reaction mixture consisted of 0.5 mL of H_2O_2 (40 mM), 2 mL of sodium phosphate buffer (50 mM, pH = 7.0), and 0.5 mL of the extracted enzyme. The drop in H_2O_2 concentration was measured for 2 minutes at a wavelength of 240 nm. One unit of enzyme activity was calculated as the quantity of µmol of H_2O_2 broken down in one minute. The POD activity was carried out according to Jiang et al. [29]. The POD reaction mixture contained 2 milliliters of buffer substrate (8 mM guaiacol, 100 mM sodium phosphate, pH 6.4), 1 mL of 24 mM H_2O_2, and 0.5 mL of enzyme extract. The amount of light absorbed was measured twice, at 30-second intervals, at a wavelength of 460 nm. POD activity was measured using the change in absorbance at 460 nm per minute per milligram of protein. The protein content was determined using the Bradford [30] technique.

2.16. Data analysis

Shapiro-Wilk test was used to test the normality of the data. Subsequently, ANOVA and the Duncan Multiple Range Test were used to compare significantly different treatment means (DMRT) using the R package "*agricolae*" [R Core Team 2022].

3. Results

3.1. Pathogen identification

ITS sequences were used to identify fungi isolated from dieback-infected xylem tissues. The sizes of the PCR products ranged from 547 to 650 bp. ITS sequences were matched to Gene Bank accessions with 77 to 99 percent similarity. The accession-like isolate #HF679024 matched *Fusarium oxysporum* at 77% of homology. Another isolate shared 99 percent of its DNA with *Curvularia lunata* to accession #MH183196.1. *Cladophialophora carrionii*, a wood-decaying fungus with the longest sequence of 650 bp, had 81 percent sequence homology with accession #LGRB01000002. *Fusarium solani* was found to be 95.42 percent identical to an isolate matched to accession # NR 163531.1. A partial homology of 85 percent sequence identity was found between *Alternaria alternata* and accession # KT699876. All sequence data can be found at the Natio-

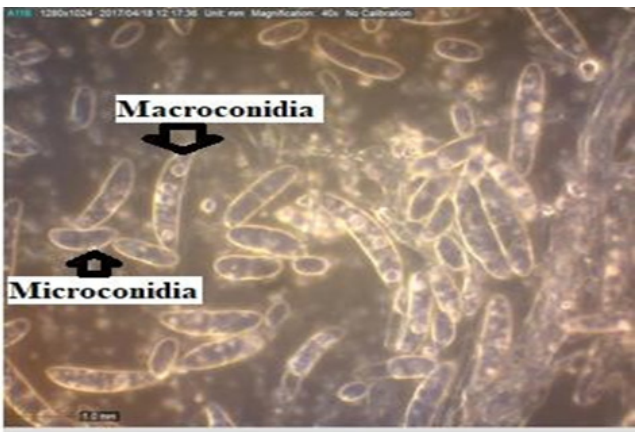

Fig. 3. Identification of *Fusarium solani* based on the morphology of micro and macroconidia.

nal Center for Biotechnology Information (NCBI) under the accession numbers MW159862.1, MW048850.1, MW048851.1, MW048852.1, and MW048852.1. The morphology of micro and macroconidia confirmed the identification of *F. solani* culture based on ITS homology (Fig. 3). Macroconidia were broad, straight, and slightly curled. They have spherical ends and 3 to 7 typical septa separations. On the other hand, microconidia were typically oval or ellipsoid in shape, with 0 to 1 septum (Fig. 3).

3.2. Pathogenicity and disease assessment trials with *Fusarium solani*

The presence of external signs such as foliar chlorosis, wilting, and stem lesions varied amongst treatments (Fig. 4). As predicted, plants under drought experienced a 90% seedling mortality rate and wilted with significant leaf chlorosis. Seedlings inoculated with *F. solani* and regularly watered showed no symptoms of dieback, and there was no significant increase in lesion size (Fig. 5). Similarly, PDA-infected plants (control) and regularly watered appeared healthy with no lesion growth and external symptoms. The growth of lesions in water-stressed seedlings inoculated concurrently or after predisposing for two weeks varied greatly (Tables 1 and 2). The external lesion measured 2.4 to 3.8 cm in length (Table 2). The maximum length of the lesion was found in seedlings predisposed to water stress (3.8 cm), followed by simultaneous (2.4 cm) and at least 0.24 cm in frequently watered seedlings (Fig. 6). *F. solani* was successfully re-isolated, and none were found in the control, confirming Koch's postulates. After

Fig. 4. Influence of *Fusarium solani* inoculation on *D. sissoo* seedlings under different watering treatments. a=Drought, b= Watered, c=Watered x *F. solani*, d= *F. solani* x Drought, e= Drought x *F. solani*.

Fig. 5. Representative images of *F. solani* lesions on *D. sissoo* seedlings subjected to various treatments. (a) Plants inoculated with PDA and regularly watered appeared healthy and free of lesions. (b) Watered seedlings inoculated with *F. solani* showed very little growth in lesion length. (c) Drought x *F. solani* inoculated seedlings and (d) predisposed to drought for 2 weeks and inoculated with *F. solani* showed a high rate of lesion growth.

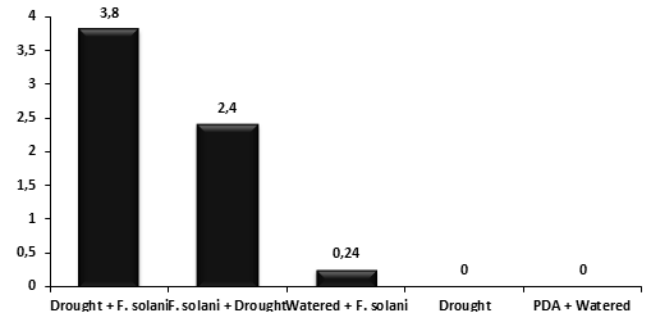

Fig. 6. Drought susceptibility before *F. solani* inoculation resulted in greater lesion growth (cm) compared to simultaneous treatment. Seedlings inoculated with F. solani and watered regularly significantly suppress pathogen growth.

Table 1. Height, lesion length, and biochemical and physiological traits of *D. sissoo* infected with *Fusarium solani* under water stress.

Parameters	Df	SS	MS	*F-value*	*P-value*
Height (inch)	4:20	130.07	32.52	58.73	9.03e-11***
Lesion growth (cm)	4:20	59.81	14.954	259.6	2e-16***
Chlorophyll *a* (µmol/ml)	4:20	1.3932	0.3483	9.806	0.000147***
Chlorophyll *b* (µmol/ml)	4:20	0.4143	0.10357	21.28	5.66e-07***
Total chlorophyll (µmol/ml)	4:20	1374.7	343.7	118.7	1.23e-13***
Carotenoids (µmol/ml)	4:20	0.06562	0.016406	13.25	1.97e-05***
H_2O_2 (U/mg)	4:20	61.27	15.318	3.581	0.0233*
CAT (U/mg)	4:20	1038.9	259.72	44.73	1.05e-09***
POD (U/mg)	4:20	61.41	15.353	7.614	0.000674***
Photosynthetic rate (µmol m^{-2} sec^{-1})	4:20	266.03	66.51	151.5	1.18e-14***
Transpiration rate (mg H_2O/m^2/sec)	4:20	13.978	3.495	283.8	2e-16 ***
Stomatal Conductance (mmol m^{-2} s^{-1})	4:20	1943599	485900	18835	2e-16***
Water Use Efficiency (kg ha^{-1} mm^{-1})	4:20	0.29024	0.07256	24.61	1.75e-07***
Osmotic Potential (MPa)	4:20	2.0760	0.5190	845.8	<2e-16 ***
Water Potential (MPa)	4:20	2.5511	0.6378	231.4	<2e-16 ***

NS. Non - significant. *p≤0.05. **p ≤ 0.01. ***p ≤ 0.001.

three years, the sissoo plants in the field appeared healthy at both locations, with no signs of dieback. Both sites were well managed by ensuring a surface water supply. Similarly, there was an increased intensity of rain during the trial period, thus ensuring plenty of water. However, three-year-old plants appeared superior in height and dbh at the Abbassia plantation than at the Cheechawatni plantation.

3.3. Evaluation of plant physiological performance

3.3.1. Plant height

Different treatments had a significant impact on seedling height. The control plants (17.42 inches) outperform saplings that were water-stressed alone or in combination with *F. solani*. The saplings subjected to water stress alone were affected the most with a plant height of (2.58 inches) or when combined with *F. solani* (Table 2).

3.3.2. Osmotic and water potential

Both osmotic and water potentials have been significantly affected by water stress (Table 1). The simultaneous treatment (-1.173 MPa) followed by drought alone resulted in a significant reduction in water potential (Fig. 7), as did seedlings predisposed to drought and inoculated with *F. solani* (-0.86 MPa) (Fig. 8). Seedlings inoculated with *F. solani* and watered daily, on the other hand, retained comparable water potentials (-0.509 MPa) to control treatments (-0.4 MPa).

3.3.3. Chlorophyll and carotenoid contents

Water stress had a significant impact on total chlorophyll, chlorophyll *a*, chlorophyll *b*, and total carotenoid content (Table 1). The concentration of chlorophyll ranged from 21.04 to 42.22 mol/ml (Table 2). When compared to controls, seedlings exposed to water stress had a significant drop in overall chlorophyll content (Table 2). Water-stressed seedlings had brown leaves at the end of the experiment, which could be attributed to a decrease in chlorophyll content, followed by predisposed saplings. The drop was more pronounced in seedlings exposed to drought alone or in combination with *F. solani* (Table 2).

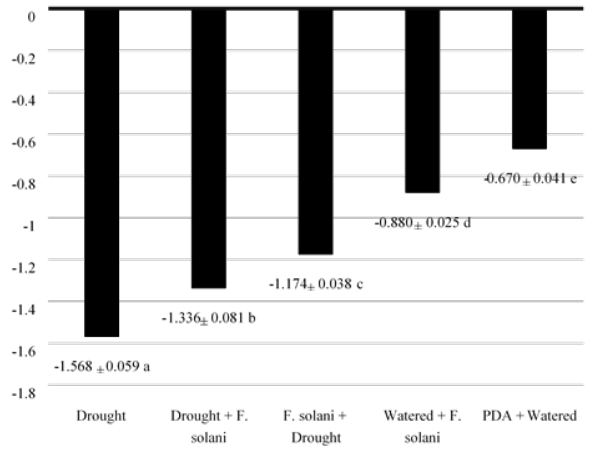

Fig. 7. Water potential (MPa) in Dalbergia sissoo plants inoculated with *Fusarium solani* concurrently with water stress imposition (F. solani + drought), drought, and control treatments (mean SD). Different letters represent statistically significant ($P < 0.05$) differences between experimental treatments.

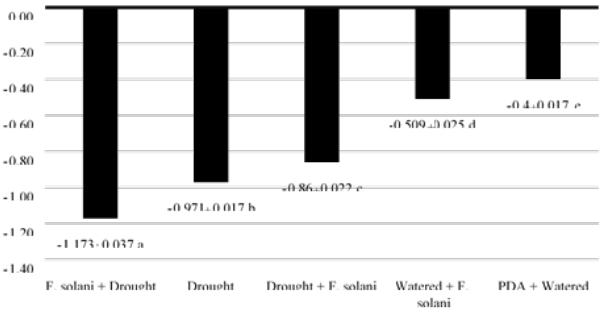

Fig. 8. Osmotic potential (MPA) in Dalbergia sissoo plants inoculated with Fusarium solani concurrently (F. solani + drought), to water stress predisposition (Drought + F. solani), and to control treatments (mean SD). Different letters represent statistically significant ($P< 0.05$) differences between experimental treatments.

Table 2. Mean comparison based on Duncan multiple range test for lesions growth, morphological and physiological parameters.

Parameters	Drought	Drought + *F. solani*	*F. solani* + drought	Watered + *F. solani*	PDA + Watered
Plant Height (inch)	2.58±1.0^{c}	8.09±1.9^{b}	5.93±2.4^{bc}	17.42±1.8^{a}	16.351.1±2.2^{a}
Lesion growth (cm)	0±0.00^{c}	3.8±0.27^{a}	2.4±0.32^{b}	0.24±0.34^{c}	c0±0.00^{c}
Osmotic Potential (MPa)	-1.173±0.037^{a}	-0.971±0.017^{b}	-0.86±0.022^{c}	-0.509±0.025^{d}	-0.4±0.017^{e}
Water Potential (MPa)	-1.568±0.059^{a}	-1.336±0.081^{b}	-1.174±0.038^{c}	-0.880±0.025^{d}	-0.670±0.041^{d}
Chlorophyll *a* (µmol/ml)	0.2±0.064^{c}	0.33±0.039^{bc}	0.416±0.04^{bc}	0.59±0.205^{b}	0.882±0.358^{a}
Chlorophyll *b* (µmol/ml)	0.064±0.044^{d}	0.226±0.055^{c}	0.264±0.04^{bc}	0.326±0.088^{b}	0.458±0.098^{a}
Total chlorophyll (µmol/ml)	21.04±2.76^{e}	26.89±1.27^{d}	32.04±0.64^{c}	36.94±1.89^{b}	42.22±1.13^{a}
Carotenoids (µmol/ml)	0.116±0.021^{c}	0.09±0.016^{c}	0.172±0.018^{b}	0.138±0.044^{bc}	0.238±0.057^{a}
H_2O_2 (U/mg)	2.204±0.697^{b}	1.822±0.596^{b}	3.934±0.718^{ab}	3.452±2.939^{ab}	6.252±3.375^{a}
CAT (U/mg)	14.368±3.08^{c}	22.57±2.88^{a}	18.148±1.32^{b}	9.714±2.63^{d}	4.056±1.62^{e}
POD (U/mg)	4.742±1.26^{a}	3.0±0.79^{ab}	4.848±2.54^{a}	1.246±0.87^{b}	1.338±0.83^{b}
Photosynthetic rate (µmol m^{-2} sec^{-1})	2.526±0.390^{d}	5.742±0.274^{c}	5.85±0.350^{c}	8.842±1.116^{b}	12.186±0.773^{a}
Transpiration rate (mg H_2O/m^2/sec)	0.3±0.48^{d}	0.776±0.65^{c}	0.828±0.35^{c}	1.72±0.14^{b}	2.382±0.18^{a}
Stomatal Conductance (mmol m^{-2} s^{-1})	29.04±0.93^{d}	44.46±2.16^{c}	42.85±2.55^{c}	585.23±9.21^{b}	629.03±5.67^{a}
Water Use Efficiency (kg ha^{-1} mm^{-1})	5.936±0.034^{a}	5.788±0.035^{b}	5.748±0.036^{bc}	5.678±0.061^{cd}	5.62±0.086^{d}

Note. Treatment means sharing the same letters, which are not significantly different.

The concentration of chlorophyll ranged from 0.2 to 0.8 mol/ml (Table 2). The highest levels of chlorophyll a were found in control seedlings, followed by inoculated and watered seedlings (Table 2). Water stressed alone (0.064 mol/ml) or in combination with fungal inoculation significantly reduced chlorophyll b levels (Table 2). A similar trend was observed for total carotenoids in saplings when water stressed alone or predisposed and inoculated showed a significant drop control (Table 2).

3.3.4. Antioxidant enzyme assay

Both water stress and pathogen inoculation had a significant effect on peroxidase (Table 2). When compared to control or inoculated and watered seedlings, stressed and inoculated seedlings had the highest POD activity (Table 2). POD activity ranged from 3.0 to 4.84 U/mg in comparison to 1.338 U/mg in control plants (Table 2). POD had a slightly different effect on CAT behavior than water and fungal stress (Table 2). High CAT expression was found in seedlings inoculated after predisposition, when both factors were simultaneously stressed, or when subjected to water stress alone. H_2O_2 levels were significantly impacted by water stress, either by itself or in combination with F. solani (Table 2). Treatments with higher CAT expression generally had lower H_2O_2 contents, suggesting a negative correlation between the two and CAT's important function in stress-induced cell damage defense against H_2O_2.

3.3.5. Stomatal conductance, photosynthetic rate, and transpiration rate

We found a statistically significant change in stomatal conductance (Table 2). In comparison to watered treatments, stomatal activity was significantly lower in water-stressed treatments (Table 2). Seedlings subjected to water-stressed (0.3 mg $H_2O/m^2/s$), predisposed (0.776 mg $H_2O/m^2/s$), and simultaneous (0.828 mg $H_2O/m^2/s$) conditions transpired at a lower rate than control seedlings (2.382 mg $H_2O/m^2/s$) due to decreased stomatal conductivity. Seedlings under water deprivation had the lowest photosynthetic activity (2.526 mol m^{-2} s^{-1}), while seedlings with ample water availability, or the control group, had the greatest (12.186 mol m^{-2} s^{-1}). Similarly, seedlings that were both inoculated and exposed to drought showed low photosynthetic activity (5.74 – 5.85 µmol m^{-2} s^{-1}), but seedlings that were both watered and inoculated showed high activity (8.84 µmol m^{-2} s^{-1}), respectively. The maximum water use efficiency was recorded by water-stressed seedlings (5.936 kg ha^{-1} mm^{-1}), which was equivalent to control and seedlings that were routinely watered and inoculated. Predisposed seedlings (5.788 kg ha^{-1} mm^{-1}) also showed a high water use efficiency during water stress and *F. solani* infection (Table 2).

4. Discussion

Molecular methods are currently extensively used to identify fungal pathogens using universal barcoding regions of the DNA. Internal Transcribed Spacer Region (ITS) based primers are universal and cover a large group of fungal taxa while at the same time having amplicons that are variable enough to efficiently distinguish between closely related species or to identify operational taxonomic units (OTUs) [31)] Based on ITS sequences, we successfully identified *Fusarium oxysporum*, *Curvularia lunata*, *Cladophialophora carrionii*, *Fusarium solani*, and *Alternaria alternate*. Most of these fungi have previously been isolated from dieback-infected *D. sissoo* plants [32-35]. Manandhar and Shrestha [36] identified *F. solani* as the primary cause of dieback, which was also reported by (30-31,33). We have observed dieback symptoms only in plants exposed to water stress. When plants are inoculated and watered regularly, they appear healthy and asymptomatic. This supports the findings of Bakhshi and Singh [37], who revealed that shisham plants were without dieback symptoms when inoculated with endophytic fungi isolated from dieback-infected plants. They also noticed that the shisham plants in the field appeared healthy during the monsoon (rainfall season with high moisture content) and showed no signs of dieback. They proposed that physiological factors such as drought, rather than fungal infections, play a primary role in sissoo dieback, with fungal pathogens acting as secondary agents. Many others have expressed similar views on the role of abiotic factors in causing shisham dieback based on their field observations [38-40]. We have further validated these findings during field surveys where sissoo trees previously infected by dieback appeared healthy due to the high number of rains received.

This is the first study to show a positive interaction between water stress and *F. solani* in causing *D. sissoo* dieback. The findings revealed that changes in morpho-physiological parameters were primarily caused by a lack of water before fungal inoculation. Furthermore, plants that were well-watered were more resistant to fungi than plants that were water-stressed. Plants inoculated after two weeks of water deprivation had more lesion growth than plants stressed and inoculated at the same time. Seedlings that had been inoculated and watered daily showed no signs of dieback or lesion formation, indicating that water stress is linked to dieback. According to Houston [41], dieback and decline are primarily caused by predisposing biotic and abiotic factors, ultimately culminating in an attack by opportunistic pathogens. Drought is probably the most important predisposing factor, with endemic facultative parasites playing a minor role. In 1998, shisham mortality reached epidemic proportions in Pakistan [7]. According to meteorological data, this outbreak was closely associated with a six-year drought that started in 1995 and ended in 2001 (Fig. 2). Precipitation amounts were 370 mm in 1995, 207 mm in 1996, 137 mm in 1997, 140 mm in 1998, 162 mm in 1999, 197 mm in 2000, and 346 mm in 2001 (Fig. 2). Mukhtar et al. [7] surveyed different ecological zones in Punjab, Pakistan, and compared the occurrence of shisham dieback in 2005 and 2009. During the 2005 survey, they reported extremely high mortality rates ranging from 6.6% to 50%, mainly due to low precipitation years. In subsequent surveys of the same regions in 2009, the disease severity decreased significantly by 2.2-8.3% because of high rainfall in 2007 and 2008 (Fig. 2). Similarly, floods, excessive rainfall, drought, and wet/dry fluctuations are all thought to be responsible for the onset of eucalypt dieback in Australia [42,43]. White [44] developed a stress index based on rainfall disturbances (wet weather followed by abnormally dry weather) and found that dieback is caused by drowning of vegetative roots during heavy rains and due to reduced root-to-canopy ratio during droughts. Using meteorological data, they found a strong positive relationship between stress indices and eucalyptus mortality. Similarly, extreme moisture fluctua-

tions such as inundation and flooding are associated with frequent dieback in shisham [35,45]. *D. sissoo* develops a complex taproot system that allows the tree to absorb water from the subsoil and grow in semi-arid conditions. Flooding, semi-flooding, and drought can all injure or destroy roots, rendering them more vulnerable to secondary fungal diseases [45]. There was a substantial difference in the morphophysiological state of whether plants were infected and stressed simultaneously or predisposed. Higher degrees of lesion development, plant growth rate, and biochemical reaction demonstrate that water stress timing and shisham physiological condition impact host susceptibility to infection [46-48]. Water stress has a major influence on photosynthesis, transpiration, and stomatal activity, whether alone or in conjunction with pathogen inoculation. All treatments, except being well-watered, lowered stomatal conductance. Drought and pathogen inoculation lowered overall chlorophyll content, chlorophyll a and b, and carotenoids in *sissoo* substantially. The biggest drop seen might be attributable to plant systems' slower metabolic rate and breakdown [49]. Another explanation for the reduction in chlorophyll and carotenoids is that the chloroplasts have been damaged excessively [50]. Reduced stomatal conductance or metabolic inhibition appears to be the reason for the observed reduction in photosynthetic rate [47-51].

The first response of plants to drought stress is stomatal closure due to water stress-induced suppression, which reduces leaf CO_2 diffusion and, thus, photosynthesis [52,53]. Under normal growing conditions, plants use energy for cell maintenance, growth, protection, and reproduction. To survive under severe conditions such as fungal infections or water stress, plants must balance energy generation and plant defenses [54,55]. Pathogen infection reduces photosynthetic activity, forcing energy resources away from growth and towards defense [56]. Our findings confirm this trade-off, which reveals that poorer plant growth arises from reduced photosynthetic activity caused by stomatal closure and chlorophyll pigment loss. Atkinson [57] demonstrated how drought affects the physiology of *D. latifolia* and *D. sissoo*, claiming that stomatal closure is the primary source of drought-induced photosynthetic suppression. Drought stress affects both species' photosynthetic rates due to stomatal and non-stomatal restrictions, as well as photoinhibition. Higher leaf water potential values may indicate lower photosynthesis, stomatal conductance, and transpiration to reduce water loss or pathogen-induced physical alterations in plant cell wall components [58]. *F. solani*, a fungus containing cell wall-degrading enzymes, can reduce cell wall flexibility while maintaining cell turgidity by lowering water potential [59]. The low water potential values reported following inoculation in vulnerable shisham plants may facilitate fungal colonization. This was also noted in *Botryosphaeria dothidea*, where infectivity increased as the plant's water potential decreased, according to Crist and Schoeneweiss [60].

The dynamic process of plant-pathogen interactions is studied, where two species, the pathogen and the host, compete to get past barriers that act as barriers. Even when far from the inoculation site, plants are still able to recognize and activate their defense mechanisms [57-58]. One of the first lines of defense against biotic and abiotic stress is the generation of reactive oxygen species (ROS). ROS generates redox imbalance and oxidative stress, which, while harmful to invaders, may impede the normal functioning of host cells [61-62]. Host plants provide enzymatic and non-enzymatic antioxidants during oxidative bursts to protect cells from oxidative stress [63]. As a pathogenicity mechanism, fungi produce antioxidant enzymes such as catalase and peroxidase [64]. Reduced H_2O_2 levels have been found in plants inoculated under water scarcity because of the ROS scavenge fungus releasing antioxidant enzymes such as CAT, which increases colonization success. Drought induces hydrogen peroxide (H_2O_2) buildup in Austrian pine (*Pinus nigra*) shoots, whereas canker pathogen *D. sapinea* significantly reduces H_2O_2 levels in water-deprived plants [44]. In fungal and water-stressed plants, CAT growth was boosted, suggesting the plant's natural defensive response. CAT is a key enzyme in plants that scavenges and decomposes H_2O_2 during oxidative stress, keeping the redox equilibrium stable [65]. Plant defense systems against pathogens are also connected to peroxidase (POD). When compared to drought-exposed or predisposition-inoculated sissoo seedlings, normal plants exhibited very low POD levels. In plants, POD's principal role is to remove H_2O_2 from tissues and contribute to the synthesis of phenolic compounds like lignin [66,67]. Peroxidase is also involved in ethylene production, and its activity rises often during pathogen infection [68,69].

Dalbergia sissoo seeds were collected from the center of origin, where shisham dieback was not observed. There was a significant ecological difference between the climate of native and non–native regions. The shisham evolved under high water requirements, absence of salinity, drought, and waterlogged conditions. *D. sissoo* was exposed to semi-arid, low rainfall, waterlogging, salinity, and frequent and prolonged droughts in non-native ecological regions. These abiotic stresses predisposed sissoo plantations to biotic factors such as fungi [1]. Shisham dieback was reported from all the plantations except for the Abbassia plantation situated in Bahawalpur, a southernmost city with a desert-like climate that had reported the lowest dieback incidence [7]. The main factor that set it apart from other plantations was the Melsi link canal that runs through the plantation to ensure a continuous supply of water and, at the same time, helps maintain the water table to mitigate evapotranspiration losses. On the other hand, large-scale shisham mortality has been observed from the Changa Manga and Cheechawatni plantations. These plantations have water available only for two to three months of the year. This resulted in water shortage and the lowering of the water table during the extreme drought period, resulting in large-scale shisham mortality. Bakhshi et al. [70] found similar results in a 110-ha sissoo plantation raised in Karnal Forest Division (Haryana) from 1952 to 1960. Irrigation was discontinued in 9 of the 11 coupes, and dieback was developed in water-stressed coupes, whereas 2 coupes that were regularly irrigated remained disease-free. They concluded that the disease was attributed to a lack of water, where trees became dry in areas of low rainfall in Haryana, where surface roots were unable to draw water from the low water table.

5. Conclusion

The findings highlight the importance of studying the relationship between biotic and abiotic variables and *D. sissoo* productivity, particularly given the present climate change scenario, which anticipates greater drought and pa-

thogen attacks. When *D. sissoo* was inoculated during water stress, its susceptibility to fungal infection increased, suggesting that early water stress reduces the host's basal defenses and allows the pathogen to invade host defenses. Water stress predominantly changes the physiological state of the sissoo through fungal infection, increasing the host's susceptibility to dieback. The complex relationship between drought and disease described here emphasizes the importance of using integrated approaches to study multiple stress variables, both biotic and abiotic, to fully understand and validate what is happening in the field. It is recommended that the genotypes that are tolerant to abiotic stress must be developed for long-term management of the shisham plantations. Secondly, shisham plantations must be established in areas that are not prone to long-term droughts, waterlogging, and salinity.

Conflict of interest
The authors declare no conflict of interest.

Ethical approval
All authors agreed on the publication, and no animal or human specimens were used or harmed in the conduct of this study.

Author contributions
MAJ conceptualized, performed lab analysis, and wrote the main manuscript. MBZ and GY carried out fieldwork and performed the collection of data. MAJ, JN, and SUR reviewed and edited the manuscript in its present form. MAJ, ZD, and SUR supervised provided funding and approved the manuscript. All authors contributed to the article and approved the submitted version.

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Availability of data and materials
Fungal sequences are available from the NCBI Gene Bank database under accession ≠ MW357979.1, MW159862.1, MW048850.1, MW048851.1, MW048852.1, and MW048853.1.

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